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Regulation of Adipocyte 11 β -Hydroxysteroid Dehydrogenase Type 1 (11 β -HSD1) by CCAAT/Enhancer-Binding Protein (C/EBP) β Isoforms, LIP and LAP

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Abstract

11 β -hydroxysteroid dehydrogenase type 1 (11 β -HSD1) catalyses intracellular regeneration of active glucocorticoids, notably in liver and adipose tissue. 11 β -HSD1 is increased selectively in adipose tissue in human obesity, a change implicated in the pathogenesis of metabolic syndrome. With high fat (HF)-feeding, adipose tissue 11 β -HSD1 is down-regulated in mice, plausibly to counteract metabolic disease. Transcription of 11 β -HSD1 is directly regulated by members of the CCAAT/enhancer binding protein (C/EBP) family. Here we show that while total C/EBP β in adipose tissue is unaltered by HF diet, the ratio of the C/EBP β isoforms liver-enriched inhibitor protein (LIP) and liver-enriched activator protein (LAP) (C/EBP β -LIP:LAP) is increased in subcutaneous adipose. This may cause changes in 11 β -HSD1 expression since genetically modified C/EBP $\beta^{(+/-)}$ mice, with increased C/EBP β -LIP:LAP ratio, have decreased subcutaneous adipose 11 β -HSD1 mRNA levels, whereas C/EBP $\beta^{\Delta uORF}$ mice, with decreased C/EBP β -LIP:LAP ratio, show increased subcutaneous adipose 11 β -HSD1. C/EBP β -LIP:LAP ratio is regulated by endoplasmic reticulum (ER) stress and mTOR signalling, both of which are altered in obesity. In 3T3-L1 adipocytes, 11 β -HSD1 mRNA levels were down-regulated following induction of ER stress by tunicamycin but were up-regulated following inhibition of mTOR by rapamycin. These data point to a central role for C/EBP β and its processing to LIP and LAP in transcriptional regulation of 11 β -HSD1 in adipose tissue. Down-regulation of 11 β -HSD1 by increased C/EBP β -LIP:LAP in adipocytes may be part of a nutrient-sensing mechanism counteracting nutritional stress generated by HF diet.

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Introduction

11 β -hydroxysteroid dehydrogenase type 1 (11 β -HSD1) is highly expressed in liver and adipose tissue where it catalyses the regeneration of active glucocorticoids (corticosterone, cortisol) from inert 11keto- forms (11-dehydrocorticosterone, cortisone) thus increasing intracellular glucocorticoid action [1]. 11 β -HSD1 expression is elevated selectively in adipose tissue of obese humans and in monogenic rodent genetic obesity, whereas levels in liver are unaffected or even decreased [2,3,4]. Transgenic over-expression of 11 β -HSD1 in adipose tissue recapitulates the metabolic syndrome in mice, with visceral obesity, dyslipidemia, insulin resistance/diabetes and hypertension [2,5]. In contrast, 11 β -HSD1-deficiency or inhibition causes insulin-sensitization (including in humans), lowers fasting plasma glucose and lipid levels, reduces visceral adipose tissue mass and attenuates atherosclerosis [6,7,8]. Unexpectedly, high fat (HF) diet down-regulated 11 β -HSD1 selectively in adipose tissue in mice and rats [9,10,11]. This down-regulation is greatest in obesity-resistant strains [9] suggesting it may be a mechanism to minimise metabolic disease with adiposity. Understanding the mechanisms

of adipose-specific control of 11 β -HSD1 is crucial to dissecting the pathogenesis of sensitivity/resistance to obesity.

Transcription of 11 β -HSD1 is directly regulated by members of the CCAAT/enhancer binding protein (C/EBP) family of transcription factors in all tissues and cells studied [12,13,14,15,16]. The family comprises 6 members; C/EBP α , β , δ , γ , ϵ and ζ (or CHOP) [17]. C/EBP α , β , δ and CHOP are essential for adipocyte differentiation and function *in vitro* and *in vivo* [18,19,20,21]. C/EBP α and β each occur as distinct isoforms arising from differential translation initiation or proteolysis [22,23,24]. C/EBP α produces 42 kDa (p42) and 30 kDa (p30) isoforms [22,24,25]. C/EBP β exists as three major isoforms; the 38 kDa and 35 kDa liver-enriched activator protein isoforms (LAP* and LAP, respectively), which stimulate transcription, and the 20 kDa liver-enriched inhibitor protein (LIP) [24]. LIP lacks the transcription activation domain of C/EBP β and is typically a dominant-negative regulator of C/EBP function [24]. The LIP:LAP ratio is thus an important determinant of C/EBP β action [26,27,28].

In vivo, C/EBP α is the major known inducer of 11 β -HSD1 transcription in liver, where C/EBP β acts as a relative repressor

[12]. In contrast, in adipose tissue, C/EBP β is an activator [29]. Moreover, C/EBP β is required for glucocorticoid-induction of 11 β -HSD1 in A549 cells [16] as well as regulation by IL-1 β , cAMP and ceramide in fibroblasts and 3T3-L1 preadipocytes [13,14,30]. However, the crucial transcriptional regulation of 11 β -HSD1 in adipose tissue and mature adipocytes remains unexplored. Here, we have tested the hypothesis that C/EBPs are modulated by diet in mice and mediate the regulation of 11 β -HSD1 in adipose.

Materials and Methods

Animals

C57BL/6J mice (Harlan UK Ltd., Oxon, UK) were housed in standard conditions on a 12 h light, 12 h dark cycle (lights on at 0700 h) at 21 \pm 1°C. Adult male mice (n = 16/group) were fed control diet (11% calories as fat; diet D12328, Research Diets, Inc., New Brunswick, NJ) or HF diet (58% calories as fat; diet D123331, Research Diets) for 6 weeks. Adult male C/EBP β mutant mice, heterozygous C/EBP $\beta^{+/L}$ and homozygous C/EBP $\beta^{\Delta\text{ORF}}$ and respective wild-type (WT) littermate control mice (n = 6–7/group) were generated as previously described [31,32] and fed standard chow diet. All animal experiments were conducted in strict accord with accepted standards of humane animal care under the auspices of the Animal (Scientific Procedures) Act UK 1986 and following prior approval by the Home Office in UK or following prior approval by the Institutional Animal Care and Use Committee in Berlin, Germany.

3T3-L1 Adipocyte Differentiation and Transfection with siRNA

3T3-L1 murine preadipocytes (ATCC, American Type Culture Collection) were maintained and differentiated into mature adipocytes as previously described [33]. Briefly, 2d after reaching confluence, 3T3-L1 cells were induced to differentiate by the addition of 0.5 μ M dexamethasone, 500 μ M 3-isobutyl-1-methylxanthine and 5 μ g/ml insulin for 2d. Thereafter, 3T3-L1 cell differentiation continued in medium supplemented with 5 μ g/ml insulin alone. Experiments were performed 8–12d following induction of differentiation, unless otherwise stated. 3T3-L1 adipocytes were transfected with siRNA using DeliverX Plus (Panomics Inc., Fremont, CA) according to the manufacturer's protocol. Fully differentiated adipocytes were gently detached by a brief wash with trypsin followed by collagenase type I (0.5 mg/ml; Invitrogen, Paisley, UK), and reseeded at 1.5 \times 10⁵ cells per well in a 12-well plate then transfected the next day with siRNA (32 pmol) in serum-free medium for 3 h followed by 21 h incubation in growth medium. All siRNAs were purchased from Applied Biosystems (Warrington, UK) and were scrambled (AM4611, negative control), C/EBP α (ID 101889), C/EBP β (ID 288793) and CHOP (ID 288792). In the experiments designed to induce ER stress, 3T3-L1 adipocytes were treated with tunicamycin (4 μ g/ml). Rapamycin (100 nM and 500 nM, 24 h) was used to inhibit the mammalian target of rapamycin (mTOR) in 3T3-L1 adipocytes.

RNA Extraction and Analysis

Following dissection, subcutaneous adipose tissue (inguinal depot) was frozen on dry ice and stored at –70°C. Tissues were weighed on a balance model AA-160 (Denver Instrument Company, NY). RNA was extracted using Qiagen RNeasy Lipid Tissue Mini kit (Qiagen, West Sussex, UK). 3T3-L1 cells were harvested in Trizol. RNA (2 μ g) was reverse transcribed using SuperScript III (Invitrogen) and quantified by real-time PCR on a LightCycler 480 (Roche) as described [16]. Primer-probe sets were purchased from Applied Biosystems: 11 β -HSD1 (Mm00476182_m1), C/EBP α

(Mm00514283_s1), C/EBP β (Mm00843434_s1), C/EBP δ (Mm00786711_s1) and CHOP (Mm00492097_m1). Routinely, at least two internal standards were used from among 18S (Hs99999901_s1), β -actin (Mm00607939_s1) and TATA binding protein (TBP; Mm00446973_m1).

Western Blot Analysis

Subcutaneous adipose tissue (inguinal depot) was homogenized in lysis buffer (0.125M Tris-HCL, pH 6.8, 2% sodium dodecyl sulphate, and 10% glycerol) in the presence of a protease inhibitor cocktail (P2714; Sigma-Aldrich, Dorset, UK) and heated at 100°C. 3T3-L1 adipocytes were harvested directly into lysis buffer and processed similarly. Electrophoresis was carried out on 4–12% NuPage Bis-Tris gels (Invitrogen). After transfer, blots were probed with antibodies to C/EBP α , - β , - δ (1:500 dilution of stock 200 μ g/ml; Santa Cruz Biotechnology Inc., Santa Cruz, CA), CHOP (1:1000 dilution; Abcam, Cambridge, UK) and β -tubulin (1:10000 dilution; Chemicon/Millipore, Watford, UK) and secondary antibodies anti-rabbit IgG-HRP (1:2000 dilution from stock 400 μ g/ml Santa Cruz Biotechnology Inc.), anti-mouse IgG-HRP (1:4000 dilution; Cell signaling, Danvers, MA), anti-rabbit IgG Alexa Fluor 700 (Invitrogen) and anti-mouse IgG (Rockland Immunochemicals Inc. Gilbertsville, PA). The resulting bands were analyzed by ImageJ (NIH, USA) or the Odyssey Infrared Imaging System (LI-COR Biosciences Ltd, Cambridge, UK), as appropriate.

Chromatin Immunoprecipitation (ChIP) Assays

ChIP assays were carried out using an Upstate EZ ChIP kit (Millipore, Billerica, MA) according to the manufacturer's protocol. Briefly, differentiating 3T3-L1 cells (8 h after induction of differentiation) or fully mature adipocytes (differentiated for 9d) in 15 cm tissue culture dishes were incubated with dimethyl adipimidate.2 HCL (10 mM in PBS; Thermo Fisher Scientific Cramlington, UK) for 30 min in the dark followed by 1% (v/v) formaldehyde. Cells were lysed in sodium dodecyl sulphate buffer (provided in the kit). Chromatin was sheared by sonication using a Soniprep 150 (MSE; Beckenham, Kent, UK) with eight 10 s pulses, at maximum amplitude, keeping cells on ice between pulses. Immunoprecipitations were carried out with 5 μ g C/EBP or control rabbit IgG antibody (Santa Cruz). After reversal of cross-links, purified DNA was amplified by standard PCR (35 cycles of 94°C 30 s, 56°C 1 min, 72°C 1 min) or real-time PCR (95°C 5 s, 65°C 1 min) as above, using primers spanning the following C/EBP-binding sites in the 11 β -HSD1 promoter [12]: footprint FP1 and FP2 (5'-CTAGTGCTGCCTGAGACTACTCC-3' and 5'-TTTTCATAACTGCCATCAAACAG-3'), FP3 and FP4 (5'-CAGTAGGAGATGCTCAGGAACC-3' and 5'-AGAGCAACGATTGGCTTTTGG-3'), FP9 (5'-ACTTGCCCTGAGAGTCTTGAAGT-3' and 5'-AGCAAAATAAGCTCAAGGGAAAA-3') and a C/EBP β binding site in the C/EBP α promoter (5'-GGTGACTTAGAGGCTTAAAGGAG-3' and 5'-GTGACTTTTCCAAGGCGGTGAGT-3'). Primers spanning an 11 β -HSD1 intronic sequence not predicted to bind C/EBP were used as a control (5'-TCAAACATC-CAGGTTCTTTTCATT-3' and 5'-CATACCACATAAACCA-CACATGG-3'). Data are expressed relative to levels of input DNA in immunoprecipitations.

Statistical Analysis

All data were analyzed by Student's *t* test or ANOVA followed by *post hoc* Tukey, Fisher LSD or Dunnett tests using SigmaStat 2.03 statistical software. Significance was set at $p \leq 0.05$.

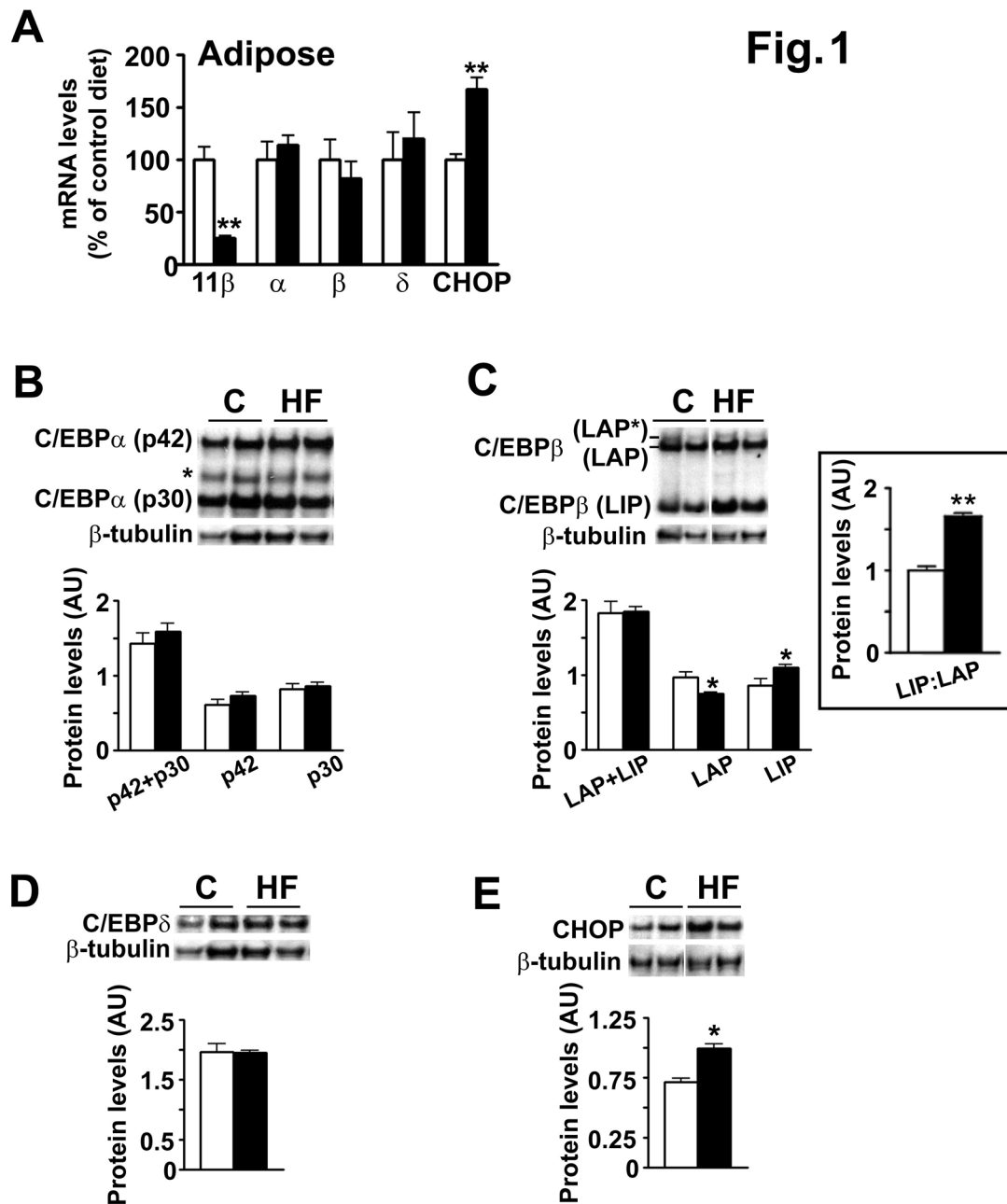


Figure 1. Expression of 11 β -HSD1, C/EBP α , β , δ and CHOP in adipose tissue of HF diet-fed mice compared to control diet-fed mice. (A) Real-time PCR measurements of levels of mRNA encoding 11 β -HSD1, C/EBP α , β , δ , and CHOP in adipose tissue of HF-fed (black bars) and control diet-fed (white bars) mice. Levels of mRNA, normalized to TBP, are expressed relative to levels in control diet-fed mice, arbitrarily set to 100%. (B-E) Representative western blots (50 μ g protein/lane) and quantification of blots probed with; (B) C/EBP α antibody showing levels of the 42 kDa (p42) and 30 kDa (p30) isoforms of C/EBP α as well as total C/EBP α (p42+p30) levels (the intermediate immunoreactive protein indicated by * is likely to be a proteolytic product of p42); (C) C/EBP β antibody showing levels of LAP (38 kDa LAP*+35 kDa LAP) and the 20 kDa LIP isoforms of C/EBP β as well as total C/EBP β (LAP+LIP) levels (inset shows LIP:LAP ratio); (D) C/EBP δ antibody and (E) CHOP antibody showing levels of respective proteins in adipose tissue of mice fed HF diet (HF, black bars) or control diet (C, white bars). Blots were stripped and reprobed with β -tubulin antibody, as loading control. In C/EBP β and CHOP westerns, all samples were analysed in the same gel but not all in adjacent lanes. C/EBP levels were quantified relative to β -tubulin levels using ImageJ analysis and are expressed in arbitrary units (AU). All values are mean \pm SEM; n = 16/group (A) or 4/group (B-E). *, $p \leq 0.05$; **, $p < 0.001$. doi:10.1371/journal.pone.0037953.g001

Results

Effect of HF Diet on 11 β -HSD1 and C/EBP Expression in Mouse Adipose Tissue

Mice fed HF diet for 6 weeks were heavier (HF, 30.8 ± 0.52 vs control diet, 26.3 ± 0.34 g; $p < 0.01$) with increased subcutaneous

adipose tissue weight (HF, 0.022 ± 0.001 vs control, 0.012 ± 0.001 (w/w) corrected for body weight; $p < 0.01$), while liver weight was unchanged (HF, 0.049 ± 0.001 vs control, 0.046 ± 0.003 (w/w) corrected for body weight). 11 β -HSD1 mRNA was down-regulated in adipose tissue by HF diet both in subcutaneous (Fig. 1A) and visceral (mesenteric) depots (data not shown), but

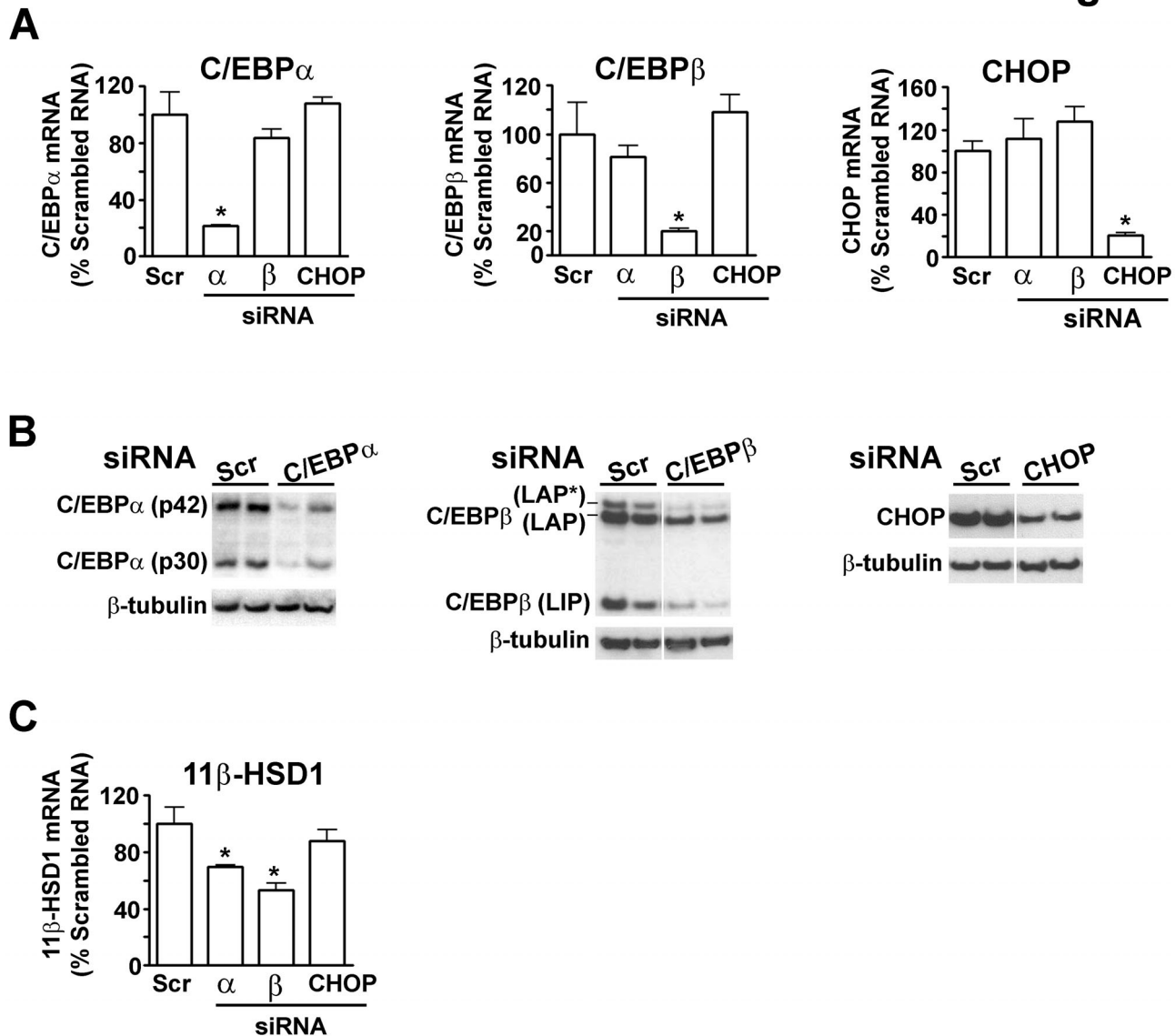
Fig. 2

Figure 2. Transfection of fully differentiated 3T3-L1 adipocytes with C/EBP α or C/EBP β siRNA decreases 11 β -HSD1 mRNA levels, whilst CHOP siRNA has no effect. (A) Real-time PCR measurement of levels of mRNA encoding C/EBP α (left panel), C/EBP β (centre panel) and CHOP (right panel), 24 h after transfection of fully differentiated 3T3-L1 adipocytes with scrambled RNA (Scr; as control) or siRNAs (32 pmol) targeting C/EBP α , β or CHOP. Data are normalized to TBP and expressed relative to levels in cells transfected with scrambled RNA (arbitrarily set to 100%). Values are mean \pm SEM of 3 different experiments (independent adipocyte differentiations) with each siRNA treatment tested in triplicate. *, Significantly different from scrambled RNA, $p \leq 0.05$. (B) Representative western blots showing levels of the 42 kDa (p42) and 30 kDa (p30) isoforms of C/EBP α (left panel; 20 μ g protein/lane), 38 kDa LAP*, 35 kDa LAP and 20 kDa LIP isoforms of C/EBP β (centre panel; 40 μ g protein/lane) and CHOP (right panel; 20 μ g protein/lane), 24 h after transfection of 3T3-L1 adipocytes with scrambled RNA or respective siRNAs targeting C/EBP α , β or CHOP. Blots were stripped and reprobed with β -tubulin antibody, as loading control. In C/EBP β and CHOP westerns all samples were analysed in the same gel but not all in adjacent lanes. (C) Real-time PCR measurement of mRNA encoding 11 β -HSD1, 24 h after transfection of 3T3-L1 mature adipocytes with scrambled RNA or siRNA (32 pmol) targeting C/EBP α , β or CHOP. Data are normalized to TBP and expressed relative to levels in cells transfected with scrambled RNA (arbitrarily set to 100%). Values are mean \pm SEM of 3 to 4 different experiments (independent adipocyte differentiations) with each siRNA treatment tested in triplicate. *, Significantly different from scrambled RNA, $p \leq 0.05$. doi:10.1371/journal.pone.0037953.g002

was unchanged in liver (data not shown), consistent with previous data showing down-regulation of 11 β -HSD1 mRNA and enzyme activity in adipose tissue of mice fed HF diet [9,11]. To test whether altered C/EBP expression may underlie the dietary regulation of 11 β -HSD1, we examined C/EBP α , β , δ and CHOP expression in adipose tissue. HF diet did not change C/EBP α , β or δ mRNA levels, while CHOP mRNA

levels were increased in subcutaneous adipose tissue (Fig. 1A) and mesenteric [11] adipose tissue (data not shown). Because 11 β -HSD1 and C/EBP mRNA levels showed the same pattern of changes in both adipose depots of HF-fed (vs control) animals, subcutaneous adipose, which is more abundant, was used for subsequent analyses. Consistent with mRNA levels, western blot analysis showed an increase in CHOP protein

Fig. 3

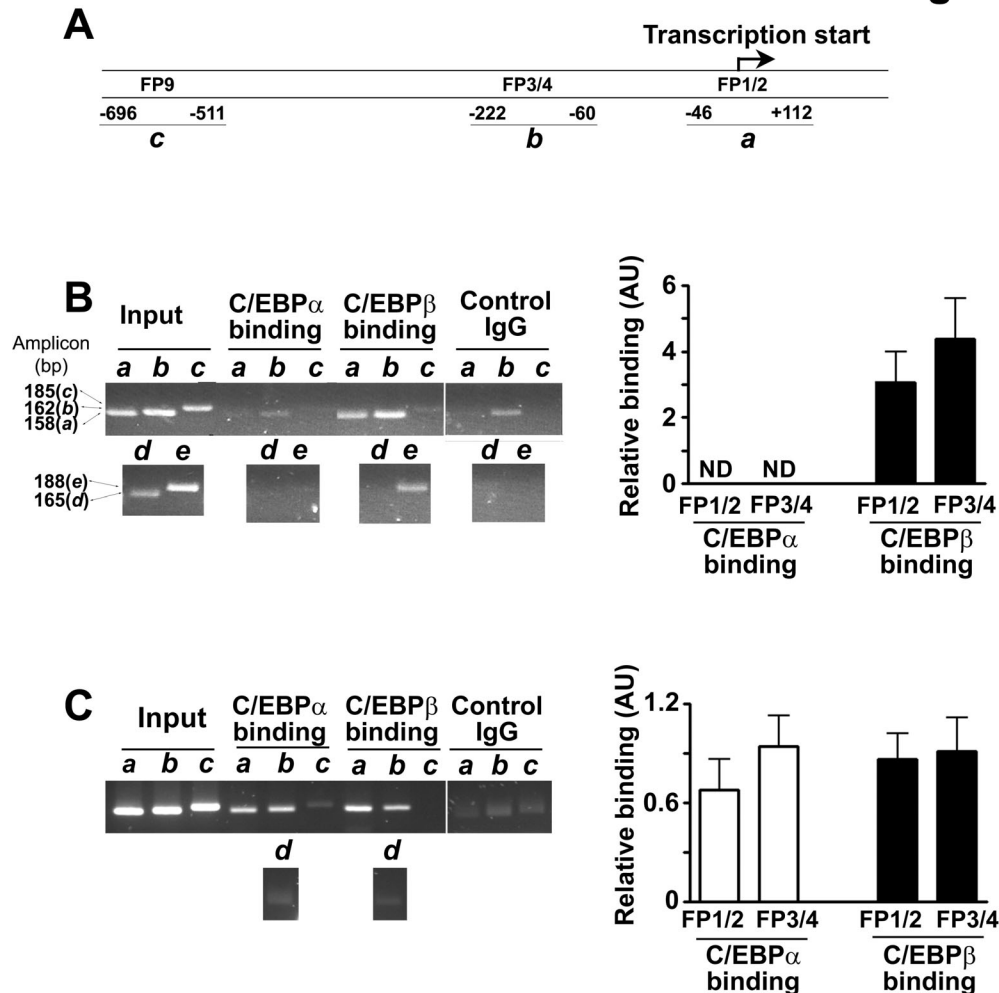


Figure 3. Binding of C/EBP α and C/EBP β to the promoter of 11 β -HSD1 throughout 3T3-L1 adipocyte differentiation. (A) Relevant C/EBP binding sites in the mouse 11 β -HSD1 promoter; FP1 to 4 and 9. The start of transcription (+1) [12] is indicated with a bent arrow. The indicated C/EBP binding sites are highly conserved between the rat and mouse 11 β -HSD1 promoters [12,13] and are named according to the sites in the rat promoter [12]. PCR products amplified in the ChIP experiment are indicated (a to c). (B) and (C) ChIP assays carried out to examine C/EBP binding to the 11 β -HSD1 promoter in (B) differentiating 3T3-L1 adipocytes (8 h after induction of differentiation) and (C) fully differentiated adipocytes (9d after induction of differentiation). Left panels show representative gels of ChIP assays following immunoprecipitation with C/EBP α or C/EBP β antibody. Control reactions contained rabbit IgG. PCR reactions (35 cycles) were carried out on input DNA (input) or on immunoprecipitated DNA with primers spanning FP1/2 of the 11 β -HSD1 promoter (lanes marked a), FP3/4 (lanes marked b), FP9 (lanes marked c), an intronic sequence from the 11 β -HSD1 gene not predicted to bind C/EBP (lanes marked d) and a C/EBP β binding site in the C/EBP α promoter [44] (lanes marked e). In (B), all PCR products were visualized in the same gel but not in adjacent lanes, whereas in (C), all PCR reactions were performed at the same time but were visualized in different gels. Right panels show real-time PCR quantification of C/EBP α and C/EBP β binding to the FP1/2 and FP3/4 regions of the 11 β -HSD1 promoter. Reactions were performed with the same primers used for standard PCR as detailed in Methods. ND, not detected. Values are mean \pm SEM from 2 to 3 independent experiments and each sample was analysed in triplicate.

levels but no alteration in total C/EBP α (p42+ p30 isoforms), total C/EBP β (LAP* + LAP + LIP) or C/EBP δ (Fig. 1B–E) protein levels with HF diet. However, HF diet reduced adipose tissue levels of the C/EBP β -LAP*+LAP isoforms, concomitantly increasing levels of C/EBP β -LIP (Fig. 1C), resulting in a significant increase in the C/EBP β -LIP:LAP ratio (Fig. 1C, inset).

C/EBP α and C/EBP β , but not CHOP, Regulate Expression of 11 β -HSD1 in 3T3-L1 Adipocytes

Increased CHOP levels or increased C/EBP β -LIP:LAP ratio could plausibly reduce adipose 11 β -HSD1 expression. The effect

of CHOP on 11 β -HSD1 expression has not been reported. Moreover, although C/EBP β regulates 11 β -HSD1 expression during preadipocyte differentiation [13,29], any role in mature adipocytes has not been tested. To investigate the requirement for C/EBP α , C/EBP β and CHOP in 11 β -HSD1 expression in adipocytes, 3T3-L1 preadipocytes were fully differentiated into mature adipocytes, which show high endogenous expression of 11 β -HSD1 [33], and then transfected with siRNAs to decrease levels of C/EBP α , C/EBP β or CHOP. Measurement of mRNA and protein levels confirmed the reduction in expression of appropriate C/EBPs with siRNA, notably reducing all isoforms of C/EBP α and C/EBP β (Fig. 2A, B) but increasing C/EBP β -

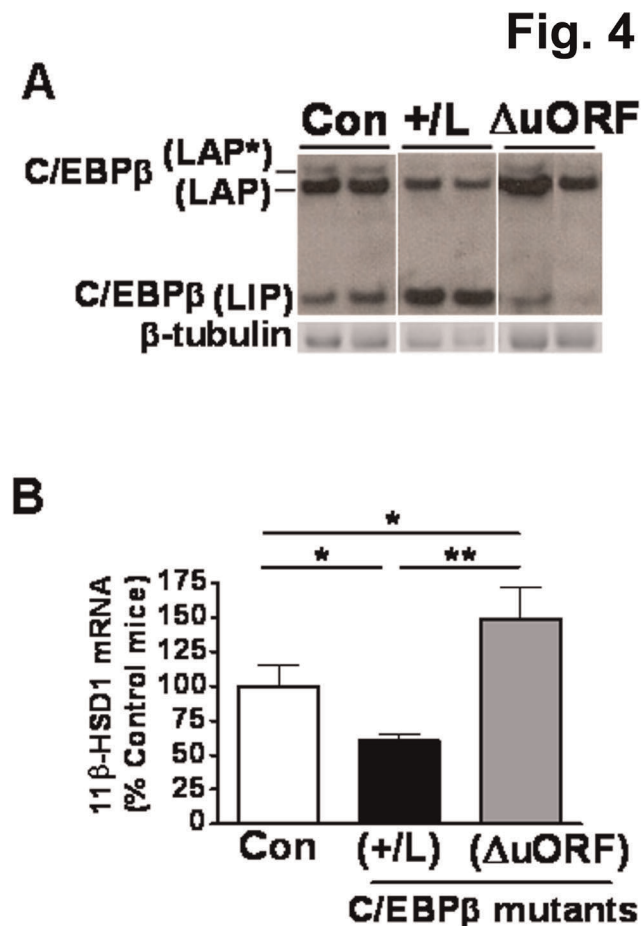


Figure 4. Altered C/EBP β -LIP:LAP ratio *in vivo* affects 11 β -HSD1 expression in adipose tissue. (A) Representative western blot (40 μ g protein/lane) showing levels of C/EBP β -LAP (38 kDa LAP* +35 kDa LAP) and -LIP (20 kDa) isoforms in the adipose tissue of C/EBP β mutants C/EBP $\beta^{+/L}$ (+/L), C/EBP $\beta^{\Delta uORF}$ (Δ uORF) and control (Con) mice. Blots were stripped and reprobed with β -tubulin antibody, as loading control. All samples were analysed in the same gel but not all in adjacent lanes. (B) Real-time PCR measurement levels of mRNA encoding 11 β -HSD1 in adipose tissue of wild type control mice (Con, white bar), C/EBP $\beta^{+/L}$ (+/L, black bar) and C/EBP $\beta^{\Delta uORF}$ (Δ uORF, grey bar). C/EBP $\beta^{+/L}$ mice are heterozygous for an allele of C/EBP β in which the normal gene has been replaced by C/EBP β -LIP (a “knock-in”) [31] and C/EBP $\beta^{\Delta uORF}$ is homozygous for the deletion of upstream ORF codon [32]. Adipose 11 β -HSD1 mRNA levels, normalized to TBP, are expressed relative to levels in control mice (arbitrarily set to 100%) and are mean \pm SEM; n = 6–9/group. *, $p \leq 0.05$; **, $p < 0.001$. doi:10.1371/journal.pone.0037953.g004

LIP:LAP ratio (scrambled RNA, 0.5 ± 0.03 vs C/EBP β siRNA 0.7 ± 0.02). The results also showed that reduced expression of one of the C/EBPs in mature 3T3-L1 adipocytes did not affect mRNA levels of the other C/EBPs tested (Fig. 2A). Both C/EBP α and C/EBP β siRNA reduced 11 β -HSD1 mRNA levels in 3T3-L1 adipocytes (Fig. 2C), demonstrating that both these members of the C/EBP family play a key role in maintaining adipocyte expression of 11 β -HSD1. In contrast, although levels of CHOP were reduced in 3T3-L1 adipocytes by siRNA (Fig. 2A, B), this did not alter 11 β -HSD1 mRNA levels (Fig. 2C).

Fig. 4

C/EBP β Locates to the Promoter of 11 β -HSD1 throughout Adipocyte Differentiation Whilst C/EBP α Binds Only in Differentiated Adipocytes

Previous reports showed C/EBP β binding to the 11 β -HSD1 promoter in undifferentiated 3T3-L1 preadipocytes [13,14], but this has not been described in adipocytes. To determine if C/EBPs interact with the 11 β -HSD1 promoter in immature and fully mature adipocytes, binding of C/EBP α and β was assayed by chromatin immunoprecipitation (ChIP). The proximal promoter of the rat 11 β -HSD1 gene contains 4 C/EBP binding sites between -196 and +44; footprints (FP) 1 to 4, which are conserved in the mouse promoter [12,13] (Fig. 3A). C/EBP β was bound to the proximal 11 β -HSD1 promoter early during adipocyte differentiation (when 11 β -HSD1 expression is low) (Fig. 3B) as well as in fully differentiated 3T3-L1 adipocytes (Fig. 3C). In contrast, C/EBP α was only bound to the 11 β -HSD1 proximal promoter in fully differentiated 3T3-L1 adipocytes, which highly express 11 β -HSD1 [33]. Neither C/EBP α nor C/EBP β were detected at FP9 in the promoter of 11 β -HSD1 (also conserved between rat and mouse) (Fig. 3B, C). Thus C/EBP β interacts directly with the 11 β -HSD1 promoter in mature adipocytes.

Increased C/EBP β -LIP:LAP Ratio *in vivo* Decreases 11 β -HSD1 mRNA Levels in Adipose Tissue, an Effect Reversed in Mice with Decreased C/EBP β -LIP:LAP Ratio

To test whether altered C/EBP β -LIP:LAP ratio can similarly alter adipose 11 β -HSD1 expression *in vivo*, we measured 11 β -HSD1 mRNA levels in subcutaneous adipose tissue of C/EBP $\beta^{+/L}$ and C/EBP $\beta^{\Delta uORF}$ mice which have increased and decreased C/EBP β -LIP:LAP ratio, respectively. In C/EBP $\beta^{+/L}$ mice, a “knock-in” of C/EBP β -LIP (L allele) replaces the normal C/EBP β gene [31], increasing C/EBP β -LIP:LAP ratio (Fig. 4A). In C/EBP $\beta^{\Delta uORF}$ mice, deletion of the upstream open reading frame (Δ uORF allele) prevents translation of C/EBP β -LIP [32], thus decreasing C/EBP β -LIP:LAP ratio (Fig. 4A). 11 β -HSD1 mRNA levels were significantly lower in adipose tissue of C/EBP $\beta^{+/L}$ mice (increased C/EBP β -LIP:LAP ratio) than in wild-type controls (Fig. 4B). The reverse was observed in C/EBP $\beta^{\Delta uORF}$ mice (Fig. 4B), demonstrating that, *in vivo*, the C/EBP β -LIP:LAP ratio controls the transcription of 11 β -HSD1.

11 β -HSD1 mRNA Levels are Down-regulated by ER Stress in 3T3-L1 Adipocytes

Elevated C/EBP β -LIP:LAP ratio and CHOP levels, observed in adipose tissue of HF-fed mice, may be a result of ER stress [34,35]. Treatment of differentiated 3T3-L1 adipocytes with tunicamycin, an inducer of ER stress [35], significantly reduced 11 β -HSD1 mRNA levels at 6 h, with a more pronounced effect at 16 h (Fig. 5A). Similar results were obtained with an alternative ER stress-inducer, thapsigargin (data not shown). Down-regulation of 11 β -HSD1 mRNA was accompanied by up-regulation of genes increased by ER stress; CHOP and the 78 kDa glucose-regulated protein (GRP78) (Fig. 5C, E), though calreticulin mRNA levels were unaffected at this stage (Fig. 5D). C/EBP β mRNA levels were not altered (Fig. 5B) whilst, as expected, C/EBP β -LIP:LAP ratio was increased, and both C/EBP β -LAP and -LIP were up-regulated (Fig. 5F). These results show that induction of ER stress, which increases the C/EBP β -LIP:LAP ratio, also down-regulates 11 β -HSD1 in 3T3-L1 adipocytes.

mTOR Regulates the Expression of 11 β -HSD1 in 3T3-L1 Adipocytes

The mTOR pathway, which is responsive to nutrient availability and growth factors, controls many aspects of cellular

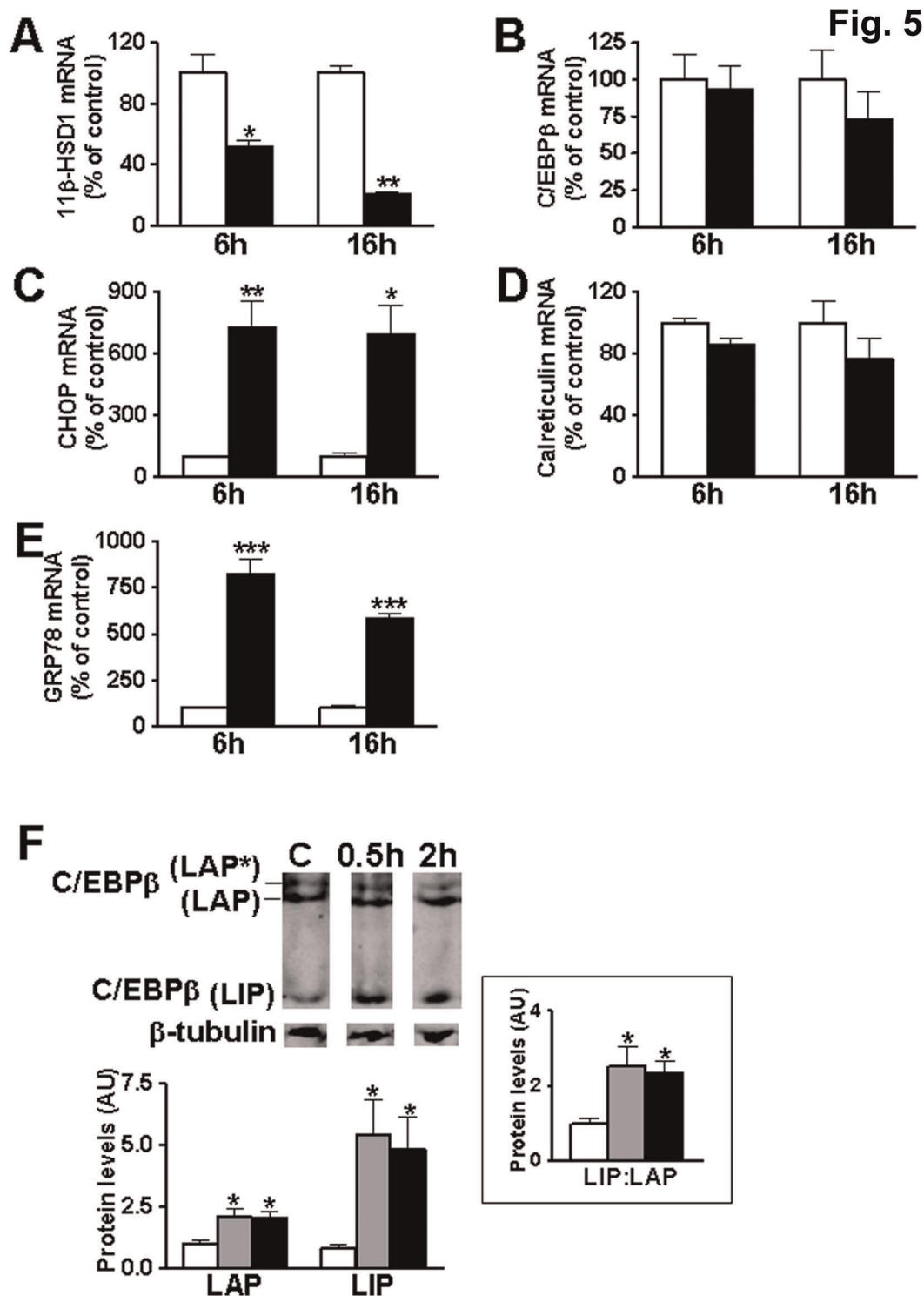


Figure 5. Tunicamycin-induced ER stress reduces 11 β -HSD1 expression in 3T3-L1 adipocytes. (A–E) Real-time PCR measurement of levels of mRNA encoding (A) 11 β -HSD1, (B) C/EBP β , (C) CHOP, (D) calreticulin and (E) GRP78 in untreated differentiated 3T3-L1 adipocytes (white bars) or treated with tunicamycin (4 μ g/ml, black bars) for 6 or 16 h. Data are normalized to TBP and expressed relative to levels of control cells (arbitrarily set to 100%). (F) Representative western blot (40 μ g protein/lane) and quantification showing levels of C/EBP β -LAP (38 kDa LAP* +35 kDa LAP) and -LIP (20 kDa) isoforms. Inset shows C/EBP β -LIP:LAP ratio in untreated 3T3-L1 adipocytes (C, white bar) or treated with tunicamycin for 0.5 h (0.5 h, grey bar) or 2 h (2 h, black bar). Blots were stripped and reprobed with β -tubulin antibody, as loading control. All samples were analysed in the same gel but not in adjacent lanes. C/EBP β levels were quantified relative to β -tubulin and are expressed in arbitrary units (AU). Values are mean \pm SEM of 2–3 independent adipocyte differentiations with each treatment tested in duplicate or triplicate. *, Significantly different from control. *, $p \leq 0.05$; **, $p < 0.001$; ***, $p < 0.0001$.

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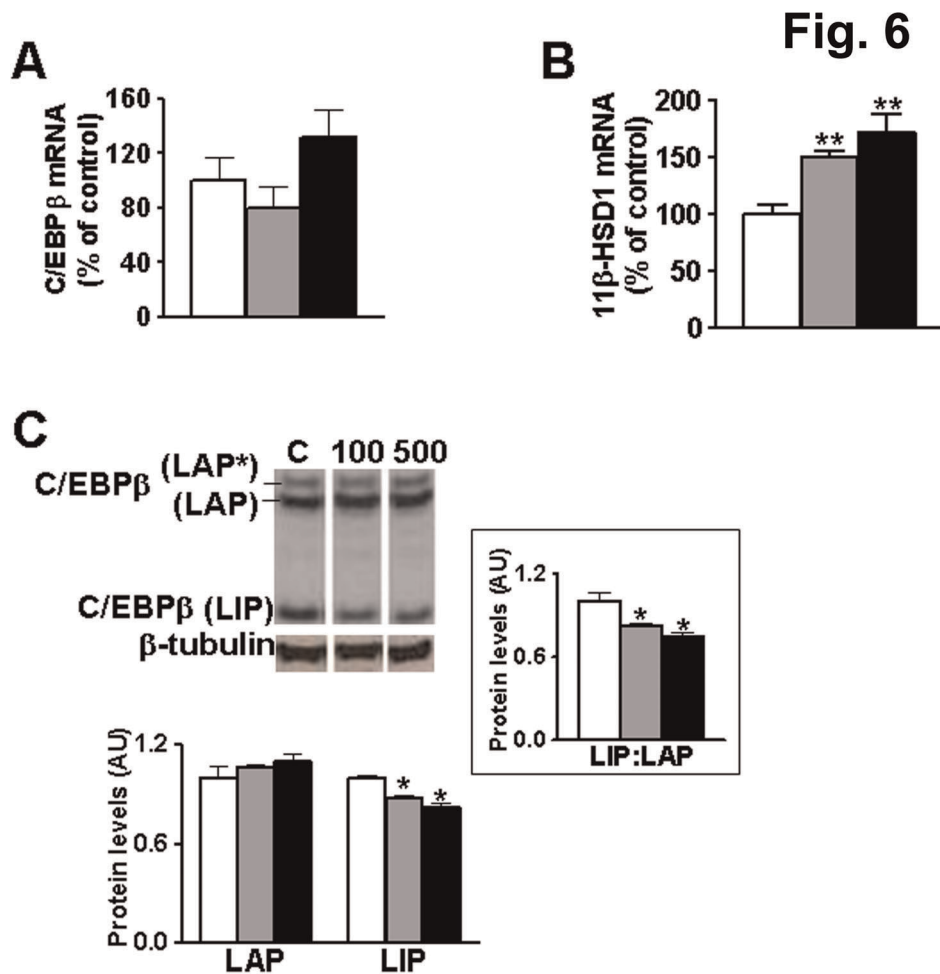


Figure 6. Inhibition of mTOR in 3T3-L1 adipocytes reduces C/EBP β -LIP:LAP ratio and increases 11 β -HSD1 expression. Real-time PCR measurement of levels of mRNA encoding (A) C/EBP β and (B) 11 β -HSD1 in 3T3-L1 adipocytes (white bars) or following treatment with 100 nM (grey bars) or 500 nM (black bars) rapamycin for 24 h. Data are normalized to TBP and expressed relative to levels of control cells (arbitrarily set to 100%). (C) Representative western blot (40 μ g protein/lane) and quantification showing levels of C/EBP β -LAP (38 kDa LAP* +35 kDa LAP) and -LIP (20 kDa) isoforms and inset showing C/EBP β -LIP:LAP ratio in untreated 3T3-L1 adipocytes (C, white bars) or following treatment with 100 nM (100, grey bars) or 500 nM (500, black bars) rapamycin for 24 h. Blots were stripped and reprobed with β -tubulin antibody, as loading control. All samples were analysed in the same gel but not in adjacent lanes. C/EBP β levels were quantified relative to β -tubulin and are expressed in arbitrary units (AU). Values are mean \pm SEM of up to 3 independent adipocyte differentiations, each treatment tested in triplicate. *, Significantly different from control. *, $p \leq 0.05$; **, $p < 0.001$.

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metabolism and regulates the production of C/EBP β -LIP and -LAP isoforms [24]. To test the importance of this pathway for the transcriptional regulation of 11 β -HSD1, rapamycin was used to inhibit mTOR in differentiated 3T3-L1 adipocytes. Rapamycin did not alter C/EBP β mRNA levels (Fig. 6A) or C/EBP β -LAP, but decreased C/EBP β -LIP, resulting in a lower C/EBP β -LIP:LAP ratio (Fig. 6C). This was associated with increased 11 β -HSD1 mRNA levels (Fig. 6B). These results show that the mTOR signalling pathway regulates 11 β -HSD1 transcription in adipocytes, plausibly via modulation of the C/EBP β -LIP:LAP ratio.

Discussion

This study emphasises the key role played by members of the C/EBP family in the regulation of 11 β -HSD1 transcription in adipocytes. However, the main finding here is that the striking down-regulation of 11 β -HSD1 in adipose tissue of mice in response to HF feeding is likely due to changes in post-transcriptional processing of C/EBP β generating an increased

C/EBP β -LIP:LAP ratio. Indeed, knock-down of C/EBP β in fully differentiated mature 3T3-L1 adipocytes showed that C/EBP β is required for normal 11 β -HSD1 expression in differentiated adipocytes, consistent with a previous report of reduced 11 β -HSD1 expression in adipose tissue of C/EBP β -deficient mice [29]. In differentiating 3T3-L1 adipocytes, C/EBP β binds to the C/EBP α promoter and induces its expression [20]. Importantly, in contrast, knock-down of C/EBP β in fully differentiated adipocytes did not affect levels of C/EBP α , suggesting a direct role for C/EBP β in adipocyte 11 β -HSD1 expression, independent of its effect on C/EBP α expression. Indeed, C/EBP α mRNA and protein levels were unchanged in adipose tissue of HF diet-fed mice. However, it cannot be excluded that C/EBP β may also exert its effect by regulating C/EBP α transactivation potential. Nevertheless, supporting a direct role, C/EBP β was bound to the 11 β -HSD1 promoter in mature adipocytes as well as in 3T3-L1 preadipocytes during the differentiation process. C/EBP α , in contrast, was only bound to the 11 β -HSD1 promoter in

differentiated adipocytes, as expected from its later appearance during differentiation [20]. Thus, both C/EBP α and C/EBP β play a direct and positive role in regulation of 11 β -HSD1 transcription in differentiated adipocytes, but C/EBP β alone is crucial in differentiating cells. This is likely to impact on the 11 β -HSD1 mRNA levels in these cells, where C/EBP α is absent or in low levels. In adipose tissue, C/EBP β and CHOP were the only C/EBPs changed by HF diet. Although CHOP generally represses transcription [36], it can also be a co-activator [37]. However, siRNA-mediated knock-down of CHOP showed that it plays no role in 11 β -HSD1 expression in fully differentiated 3T3-L1 adipocytes, although we cannot rule out that increased CHOP levels may contribute to the down-regulation of 11 β -HSD1 in adipose tissue with HF diet.

An altered C/EBP β -LIP:LAP ratio, like the HF-induced increase in CHOP, might be a response to ER stress as a consequence of a high demand for ER activity with nutrient excess [34]. The C/EBP β -LIP:LAP ratio is controlled by the double stranded RNA-dependent protein kinase (PKR, a target of ER stress [38]) and mTOR signalling pathways through the eukaryotic translation factors eIF-2 α and eIF-4E, respectively [24]. Indeed, diet-induced obesity in mice results in the activation of ER stress pathways in metabolically relevant tissues, such as adipose and liver, with increased phosphorylation of the PKR-like kinase and its substrate eIF-2 α , which are key indicators of ER stress [34]. mTOR may contribute to the ER stress response as well [39]. Our results show that both induction of ER stress and inhibition of mTOR signalling alter C/EBP β -LIP:LAP ratio and modulate 11 β -HSD1 mRNA levels in 3T3-L1 adipocytes. ER stress and mTOR signalling pathways are sensitive to cellular nutrients and energy homeostasis, suggesting that the increased C/EBP β -LIP:LAP ratio in obese adipose tissue may result from activation of these nutrient/stress sensing mechanisms leading to the down-regulation of 11 β -HSD1.

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Author Contributions

Conceived and designed the experiments: CLE KEC. Performed the experiments: CLE VK VB TYM. Analyzed the data: CLE JRS KEC. Contributed reagents/materials/analysis tools: NMM AL. Wrote the paper: CLE JRS KEC.

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